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Genetics

Early appearance and long-term persistence of the submicroscopic extrachromosomal elements (amplisomes) containing the amplified *DHFR* genes in human cell lines

(cytogenetic abnormalities/drug resistance/pulsed-field gel electrophoresis)

GIOVANNI PAULETTI, ERIC LAI*, AND GIUSEPPE ATTARDI†

Division of Biology, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **Submicroscopic extrachromosomal elements (amplisomes) containing amplified dihydrofolate reductase (*DHFR*) genes have been investigated in a methotrexate-resistant derivative of the human cell line HeLa BU25, 10B3, by field-inversion gel electrophoresis. The amount and kinetics of formation of these elements have been correlated with the level and time course of overall *DHFR* gene amplification. The amplisomes account for the great majority and possibly the totality of the amplified *DHFR* genes in 10B3 cells. They appear very early during the development of methotrexate resistance and increase in parallel with the amplified genes. These observations suggest that these elements are involved in an early event, possibly the first event, of gene amplification in this system. Amplisomes tend to be lost from 10B3 cells in the absence of selective pressure, although much more slowly than expected from simple dilution of nonreplicating elements. Surprisingly, under selective pressure, these elements have shown no tendency to become integrated into chromosomes or to generate minute chromosomes over a period of almost 1 year, in contrast to what has been described in other systems.**

Besides the previously described cytogenetic changes accompanying gene amplification (1, 2) [i.e., "extended chromosome regions" and "double minute chromosomes" (DMs)], submicroscopic extrachromosomal elements containing amplified genes have been identified. These structures were discovered in *Leishmania* species as 30-kilobase (kb) supercoiled-circular DNA molecules containing amplified copies of the gene for the bifunctional protein dihydrofolate reductase–thymidylate synthetase (3, 4). Similar but larger submicroscopic elements (120–750 kb) have been identified in mammalian cells and shown to carry amplified copies of the *DHFR* gene in methotrexate (MTX)-resistant derivatives of HeLa BU25 and VA_2B cells (5), the multiple drug resistance gene (*mdr*1) in vinblastine-resistant KB-V1 cells (6), the *MYC* gene in HL60 and COLO320 DM tumor-derived cells (7), or the transfected *CAD* gene in CHO cells (8). The submicroscopic elements are clearly distinct from the minute chromosomes; they are not only much smaller but also strikingly homogeneous in size. We propose the term "amplisomes" (i.e., bodies carrying amplified genes) to designate these submicroscopic extrachromosomal elements. This term includes the reference to gene amplification but avoids the implication that the element can alternate between integrated and free states (9), an implication that was inherent in the previous term "episome" (8) and a property that has not been shown as yet to apply to the submicroscopic elements. In the present work, we have investigated the kinetics of appearance of amplisomes in HeLa BU25-10B3 (5), correlated the kinetics of formation of the amplisomes with the process of overall *DHFR* gene amplification, and analyzed their long-term behavior in the presence or absence of selective pressure.

MATERIALS AND METHODS

Cell Lines. The human cell line HeLa BU25 and its MTX-resistant variants 10B1, 10B2, and 10B3 have been described (10). Briefly, a clonal isolate of HeLa BU25 cells adapted to grow in 90 nM DL-MTX was split into three cultures, which were then subjected to three regimens of drug administration up to 0.18 mM DL-MTX, thus producing the three 10B variants above. In addition, the 10B3 cell line was adapted to grow in suspension (referred to as 10B3S).

Preparation of DNA. Total DNA for dot-blot analysis was prepared from cell cultures by the method of Davis *et al.* (11). An automated extractor (Applied Biosystems model 340A) was used to prepare high molecular weight DNA for Southern blot analysis. The preparation of DNA in agarose blocks was performed as described (5), except for the omission of the RNase treatment and the reduction of proteinase K to 1 mg/ml. *Sal* I digestion of DNA in agarose slices was prepared according to standard procedures (3) after proteinase K inactivation by phenylmethylsulfonyl fluoride. After incubation for 1 hr with the enzyme, the slices were further incubated for 2 hr at 50°C with proteinase K at 1 mg/ml and then extensively washed in field-inversion gel electrophoresis (FIGE) buffer (see below).

FIGE. A horizontal chamber (30 × 30 cm) connected to an electromechanical relay controlled by a computer was employed for FIGE analysis (12). The 1% agarose gels (SeaKem, ME; FMC) were cast and electrophoresed at 14°C in 45 mM Tris/45 mM borate/2 mM EDTA, pH 8. A ramped-pulse regime from 3-sec to 50-sec switching cycles, with a constant 3:1 ratio between the forward and the reverse interval, was applied for 20 hr with a voltage gradient of 10 V/cm.

Southern and Dot Blot Analysis. Ethidium bromide (EtdBr)-stained gels were UV-irradiated at 254 nm for 60 sec at a flux intensity of 2 mW/cm^2. After transferring the DNA fragments to a nylon membrane (Zeta-Probe, Bio-Rad) by capillarity under alkaline conditions (13), for 2 days for FIGE gels and 16 hr for conventional agarose gels, the membranes were neutralized, washed with 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), UV-irradiated at 312 nm for 5 min, and baked at 80°C for 1 hr. For dot-blot analysis, samples of total DNA in solution or derived from agarose slices, melted and diluted in 5 vol of H_2O, were

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Abbreviations: DHFR, dihydrofolate reductase; DMs, double minute chromosomes; MTX, methotrexate; FIGE, field-inversion gel electrophoresis; EtdBr, ethidium bromide.

*Present address: Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514.
†To whom reprint requests should be addressed.

sheared by several passages through a 25-gauge needle and subjected to RNase A digestion (50 μg/ml). After incubation in 0.4 M NaOH for 2 hr at 68°C, the samples were cooled and applied onto a Zeta-Probe membrane using a commercial slot-blot apparatus (Schleicher & Schuell). As probes, the insert of the recombinant plasmid pHD84, containing a human DHFR cDNA (14), and a chromosome 1p-specific clone, pYNZ2 (15), radiolabeled by extension of random hexanucleotide primers (16), were used for high-stringency hybridization.

γ-Irradiation. The ^{60}Co single-point source at the Jet Propulsion Laboratory (California Institute of Technology, Pasadena, CA) was employed to irradiate the samples. Agarose slices were placed in wells of a 24-well microwell plate and irradiated at room temperature under conditions ensuring a uniform radiation flux to all the samples. Before and after irradiation, the samples were stored in 10 mM Tris·HCl, pH 8.0/100 mM EDTA at 10°C. FIGE fractionation was performed within 2 hr of irradiation.

RESULTS

***DHFR* Gene Copy Number of HeLa BU25 MTX-Resistant Variants.** The MTX-resistant derivative HeLa BU25-10B3, which was shown to harbor *DHFR* gene-containing amplisomes (5), and two related MTX-resistant variants, HeLa BU25-10B1 and -10B2, lacked any obvious "extended chromosomal regions" and had a small number of DMs per cell (10). Fig. 1*a* shows a Southern blot of HeLa Bu25-10B1, -10B2, and -10B3 cell DNA and, for comparison, HeLa S3 and HeLa BU25 DNA, digested with *Eco*RV and probed with pHD84. The *Eco*RV fragments containing *DHFR* gene segments are much more abundant in the MTX-resistant variants than in HeLa BU25, ≈250 times in 10B1 and 10B3 and ≈160 times in 10B2, as estimated by densitometry. These data for the relative *DHFR* gene copy number in the three MTX-resistant HeLa BU25 derivatives agree remarkably well with the previous estimates of enzyme activity in the three cell lines (10). The *Eco*RV fragments that are not amplified presumably contain *DHFR* pseudogenes or fragments thereof (17).

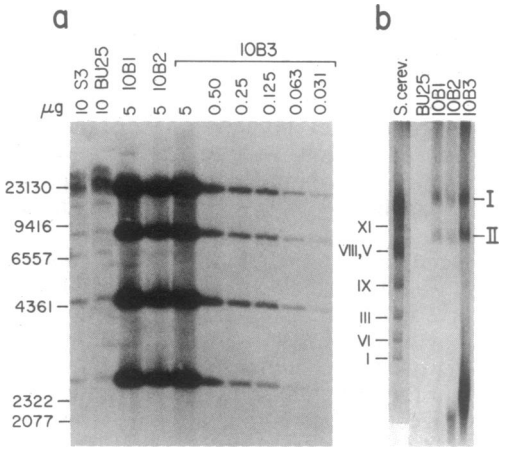

FIG. 1. Southern blot analysis of DNA from the HeLa S3, HeLa BU25, HeLa BU25-10B1, -10B2, and -10B3 cell lines, using the pHD84 probe containing human DHFR cDNA. In *a*, the indicated amounts of *Eco*RV-digested total DNA were fractionated by conventional electrophoresis in a 0.7% agarose gel; in *b*, agarose-embedded total DNA from 2 × 10^6 cells was analyzed by FIGE. The size markers of *a* were derived from a *Hin*dIII digest of λ DNA. In *b*, the roman numerals on the left indicate yeast chromosomes (*Saccharomyces cerevisiae* strain YNN295). I and II on the right of *b* indicate amplisome bands I and II.

As shown in Fig. 1*b*, fractionation of 10B1, 10B2, and 10B3 DNA by FIGE, followed by DNA-transfer hybridization with the pHD84 probe, revealed two bands of similar intensity (bands I and II). The faster moving band II corresponds to the 650-kb elements identified in 10B3 DNA (5). The mobility of these DNA molecules in pulsed-field gel electrophoresis or in FIGE is affected by the pulse time as is the mobility of linear yeast chromosomes, suggesting that the 650-kb band elements are linear (5). The slower moving band I, in turn, corresponds to another type of extrachromosomal element containing *DHFR*-specific sequences that were characterized in 10B3 DNA by a mobility that was relatively insensitive to the pulse time (5). This behavior in pulsed-field gel electrophoresis commonly indicates a supercoiled-circular DNA structure (4, 18). When rerun, most elements of band I migrated in FIGE as the 650-kb linear amplisomes (band II) (data not shown). This observation supports the proposal that the two types of elements are interrelated, representing two topological forms of the same elements or the same form in two states due to artifacts resulting from FIGE. In the present analysis, the submicroscopic elements contained in the two bands will be considered together.

Kinetics of Appearance of *DHFR* Amplisomes. As shown in Fig. 2*a*, amplisomes can be seen in 10B3 cultures resistant to 1.8 μM DL-MTX (see also below). Despite some fluctuation in the signal intensity, due to variation in cell concentration in the agarose slices of different samples (see Fig. 2), there is a trend toward an increase in the amount of amplisomes at successive stages of MTX selection with no change in size. Fig. 3 shows densitometric data for amplisome bands from cultures at various times during the selection. Data were corrected for variation in input DNA by dot-blot hybridization with the single-copy probe pYNZ2. The DNA was obtained from slices of the same agarose gel plugs prepared

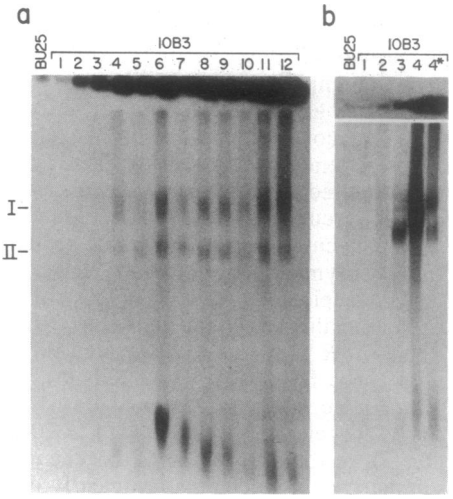

FIG. 2. FIGE analysis of the time course of appearance of *DHFR* amplisomes in HeLa BU25-10B3 cells at various stages during step-wise selection in the presence of MTX. (*a*) Total DNA from agarose-embedded cells growing at various DL-MTX concentrations. Lanes: 1, 18 μM; 2, 90 nM; 3, 1.8 μM; 4, 3.6 μM; 5, 7.2 μM; 6 and 7, 14 μM; 8, 29 μM; 9, 58 μM; 10, 0.12 mM; 11, 0.18 mM. The number of cells embedded in each slice were ≈2 × 10^6 except for lane 4 (≈4 × 10^6 cells), lane 6 (≈5 × 10^6 cells), lane 7 (≈2.5 × 10^6 cells), and lane 10 (≈8 × 10^6 cells). Roman numerals on the left indicate the upper (I) and lower (II) amplisome bands. (*b*) DNA/agarose inserts from the same preparations used for the first five lanes (BU25 sample inclusive) of the gel in *a* were γ-irradiated at 60 Gy. Lane 4* represents an unirradiated control of the sample in lane 4. The segment of the gel containing the DNA/agarose slices was excised and blotted separately to prevent diffusion of small DNA fragments (presumably single stranded, released by the alkali treatment of the UV-irradiated gel) into the upper portion of the gel during blotting.

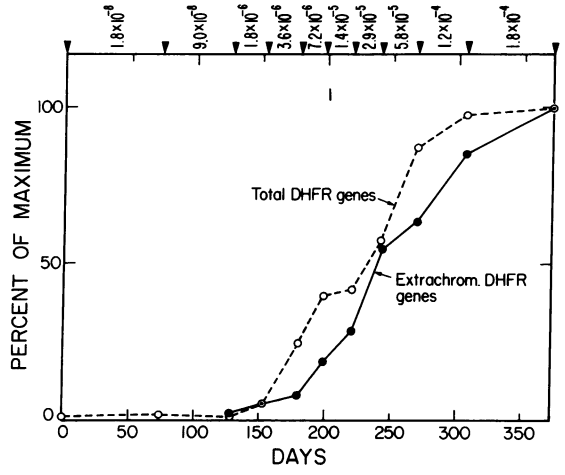

FIG. 3. Time course of increase in the amount of *DHFR* genes associated with amplisomes and with total DNA in HeLa BU25-10B3 cells during step-wise selection in the presence of MTX. The densitometric data of Southern blot and dot-blot analysis have been normalized for variation in input DNA content of the slices on the basis of hybridization with the pYNZ2 probe. The numbers at the top indicate the molar concentration of DL-MTX to which the cells were exposed, and the arrowheads indicate the time limits of exposure of the cells to each concentration.

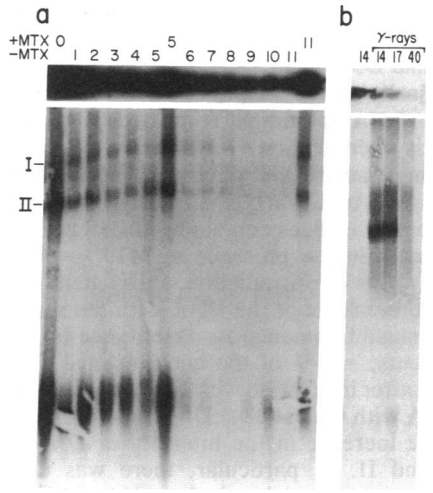

FIG. 4. Quantitative behavior of *DHFR* amplisomes in HeLa BU25-10B3 cells after removal of MTX. (*a*) DNA/agarose inserts from ≈2 × 10^6 cells were prepared at regular intervals after withdrawing the drug and subjected to FIGE. Numerals (lane labels) indicate weeks of culture without MTX (−MTX) or with MTX (+MTX). (*b*) FIGE of γ-irradiated DNA/agarose inserts prepared from cells grown for 14, 17, and 40 weeks in absence of MTX. A nonirradiated 14-week (−MTX) sample was also subjected to FIGE fractionation.

for FIGE. The data are expressed as percent of the value found for cells resistant to 0.18 mM DL-MTX. Also shown in the diagram is the total *DHFR* gene content determined by reprobing the stripped dot blots with the insert of pHD84 and normalizing the data for input DNA variation. A similar curve for the total *DHFR* gene content was determined with dot-blotted samples of total DNA extracted directly from the cell cultures. The increase in the amount of amplisomes and the increase in total *DHFR* gene number are parallel throughout the gene amplification process.

Instability of *DHFR* Amplisomes in the Absence of Selective Pressure and their Long-Term Persistence Under Selection. A culture of 10B3 cells resistant to 0.18 mM DL-MTX was grown for about 9 months in the absence of the drug, whereas a parallel culture was maintained in the presence of 0.18 mM DL-MTX. As shown in Fig. 4*a*, during growth in the absence of the drug, 10B3 cells exhibit a relatively slow loss of amplisomes (≈70% loss in ≈30 generations) for 11 weeks, after which amplisomes become barely perceptible. These data confirm previous results from this laboratory (10), showing the instability of DHFR activity in 10B3 cells in the absence of selective pressure. By contrast, over a period of 40 weeks, there is no detectable loss of amplisomes in the culture under constant selective pressure. Quantitation of the amplisomes in the two cultures normalized for variation in the level of DNA in the agarose slices (carried out as described above) is shown in Fig. 5. The curve includes data up to 40 weeks of drug withdrawal. The relative amounts of total *DHFR* genes in the DNA-containing agarose (DNA/agarose) plugs of the same cell samples are also shown for comparison. Here again, a parallel exists between loss of amplisomes and loss of total *DHFR* genes in the absence of selection. Similarly, the number of amplisomes and the total number of *DHFR* genes are unchanged over a 40-week period in the presence of MTX.

Extensive Trapping of Amplisomes at the FIGE Origin. Sucrose gradient sedimentation analysis of a metaphase chromosome preparation from 10B3 cells (5) and the absence of an appreciable number of microscopically recognizable DMs in these cells (5, 10) suggested that most of the amplified *DHFR* genes in this cell line are associated with submicroscopic elements. Therefore, the fact that only a minor fraction of *DHFR*-specific DNA sequences moved into the gel by FIGE or pulsed-field gel electrophoresis (5) indicated that the migration of the majority of these elements was prevented either by their structural features or by some form of trapping by the chromosomal DNA. In conventional agarose gel electrophoresis, relaxed-circular molecules larger than a critical size have a negligible mobility and stop at the origin, the critical size depending mainly on the field strength and the agarose concentration (19). The same phenomenon has also been observed in FIGE (20). On the other hand, trapping of the submicroscopic elements by the chromosomal DNA in the agarose block appeared to be a distinct possibility. If the operation of inverting a DNA/agarose slice and subjecting it again to FIGE is repeated several times, each time an amount of *DHFR* amplisomes equal to or greater than in the first

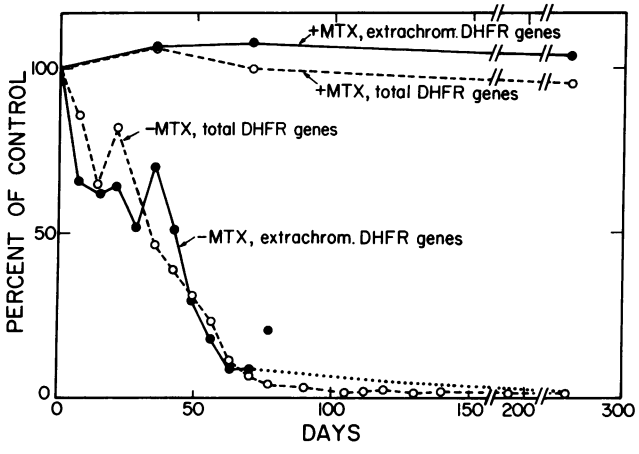

FIG. 5. Quantitative behavior of the *DHFR* genes associated with amplisomes and of total *DHFR* genes in HeLa BU25-10B3 cells grown in the absence of MTX (lower curves) or in the presence of 0.18 mM DL-MTX (upper curves). The data have been derived and normalized as explained in Fig. 3. The dotted line represents an extension of the −MTX curve for the extrachromosomal elements joining the 10-week and 40-week points (no precise quantitation was done for this portion of the curve).

FIGE migrated into the gel (data not shown). This observation is consistent with a trapping of amplisomes by the chromosomal DNA.

The Majority of the Amplified *DHFR* Genes Can Be Accounted for by Amplisomes. To provide a quantitative estimate of this phenomenon, γ-irradiation, which is known to produce single-strand and double-strand DNA breaks (21), was employed. As shown by the EtdBr-stained pattern in Fig. 6*a*, when agarose slices containing 10B3S cell DNA (grown for 10 months in the presence of MTX) were exposed to increasing doses of γ-irradiation, subsequent FIGE showed progressive breakage of the DNA with the resulting heterogeneously sized fragments. As determined by densitometric measurements, ≈95% of the chromosomal DNA migrated into the gel after irradiation with 100 Gy. By contrast, probing of the DNA with the pHD84 cDNA insert revealed a striking progressive increase in the intensity of *DHFR* amplisome bands I and II. In particular, there was no increase in pHD84-reactive material in the broad band of heterogeneous fragments containing *DHFR* gene sequences at the bottom of the gel. Only at higher doses (50 and 100 Gy) of γ-irradiation was there some evidence of a smear of material reacting with the pHD84 probe, which moved faster than the 650-kb amplisomes, suggesting the beginning of amplisome degradation. At 180 Gy and above, degradation became significant (data not shown), producing a continuous smear throughout the lane. By densitometric analysis of appropriate exposures of the autoradiogram, we estimated that the *DHFR*-specific DNA sequences associated with amplisomes at 100 Gy represented up to 400% the *DHFR*-specific sequences remaining in the DNA/agarose slices at the origin. Due to the difficulty of estimating the efficiency of transfer and hybridization of the chromosomal DNA remaining in the slices at the origin, the above quantitation can only be useful for comparing the effects of different doses of γ-irradiation.

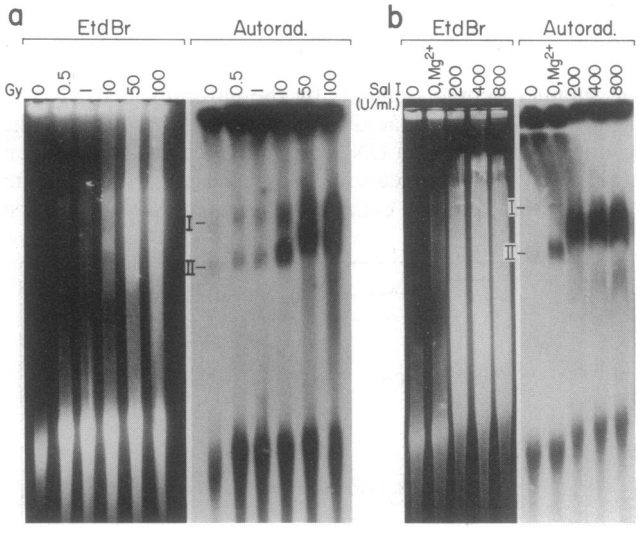

FIG. 6. γ-Irradiation or *Sal* I digestion of 10B3 DNA/agarose slices reduces trapping of amplisomes at the FIGE origin. (*a*) FIGE of 10B3 DNA/agarose inserts from ≈2 × 10^6 10B3S cells (grown in suspension for 10 months in 0.18 mM DL-MTX) subjected to increasing doses of γ-irradiation, as indicated in Grays (Gy): EtdBr-staining pattern and DNA blot probed with pHD84 (Autorad.). (*b*) FIGE of 10B3S DNA/agarose inserts (same preparation as above) subjected to *Sal* I digestion at three enzyme concentrations, as indicated in units (U)/ml. EtdBr-staining pattern and a DNA blot probed with pHD84 (Autorad.) are shown. Lanes: 0, sample pretreated as the *Sal* I-digested samples; 0, Mg^{2+}, sample pretreated and incubated at 37°C as the *Sal* I-digested samples, except without the restriction enzyme. The smear in the upper portion of the first three lanes in the autoradiogram of *b* is due to diffusion of small DNA fragments from the gel slices at the origin during blotting (see Fig. 2).

However, the results clearly show the progressive release of amplisomes from the slices by γ-irradiation.

An alternative approach for disrupting the chromosomal DNA network in the agarose slice was suggested by the finding that the restriction enzyme *Sal* I does not cut 10B3 amplisomes; thus presumably intact elements can be released from digested total DNA samples. As shown by the EtdBr-stained pattern in Fig. 6*b*, digestion of the DNA/agarose slices with high concentrations of *Sal* I prior to FIGE resulted in a massive release of heterogeneously sized chromosomal DNA fragments. It was determined that >95% of the chromosomal DNA digested by *Sal* I at 800 units/ml migrated into the gel by FIGE. In contrast to the total chromosomal DNA, the *DHFR*-specific DNA sequences released by *Sal* I digestion again migrated only with bands I and II of amplisomes, which were increased significantly in amount relative to the untreated sample. The level of intensity of these bands was approximately equivalent to that of the *DHFR* sequences left in the DNA/agarose slices after digestion with *Sal* I at 200 units/ml and remained relatively constant at higher concentrations of the enzyme. Therefore, the released amplisomes did not reach the level obtained with the highest doses of γ-irradiation. A dramatic additional release of amplisomes (≈200% of those still remaining in the DNA/agarose slice) was obtained by γ-irradiation of *Sal* I-digested DNA/agarose slices subjected once to FIGE (data not shown), confirming the much greater effectiveness of γ-irradiation in releasing the trapped amplisomes, due to the direct breakage of these structures. The closer migration of the upper and lower bands containing such structures as well as the somewhat lower mobility of the heterogeneously sized fragments containing *DHFR*-specific sequences near the bottom of the gel (phenomena already noticed after γ-irradiation) are presumably the consequence of massive amounts of chromosomal DNA in the gel. The observation that, in the control sample incubated without enzyme in Mg^{2+}-containing buffer, there was a substantial increase in the intensity of band II suggests that endogenous nucleases may have been activated, with a resulting partial digestion of the chromosomal DNA meshwork and release of linearized amplisomes.

As illustrated in Fig. 2*b*, γ-irradiation of the DNA/agarose slices from the earliest time points in the step-wise selection of 10B3 cells clearly showed a release of amplisomes that migrated with the faster moving band when the DNA sample was from cells resistant to 1.8 μM DL-MTX. There is a hint of these elements also in the sample from cells resistant to 90 nM DL-MTX and possibly also in samples from cells resistant to 18 nM DL-MTX and from the parental HeLa BU25 cells. A similar analysis (Fig. 3*b*) revealed the presence of amplisomes, migrating mainly with the lower bands, in the DNA samples from cells grown for 14 or 17 weeks in the absence of MTX.

DISCUSSION

Several main conclusions have been derived from this work. First, amplisomes could be recognized, during the step-wise selection in MTX of 10B3 cells, as early as the first detectable amplified genes. This observation is consistent with the possibility that the release of the first of these elements from the chromosomal DNA [presumably from the origin locus in chromosome 5 (17)] was, in this cell line, the initial or a very early event in the gene amplification process. Furthermore, the finding that, in cells examined at various stages of MTX resistance, the *DHFR* gene number and the amplisome number increased in parallel supports the hypothesis that the replication of amplisomes was the only or main mechanism underlying gene amplification in this system. The results of γ-irradiation or *Sal* I digestion of the DNA/agarose slices prior to FIGE also argue strongly that very few, if any, of the

DHFR genes in 10B3 cells are not associated with amplisomes. The observation that γ-irradiation is more effective than *Sal* I digestion in releasing amplisomes from the agarose slice is consistent with these elements having a circular structure. The elements that remained trapped in the gel slices after the most extensive γ-irradiation or *Sal* I digestion may represent circular molecules that became nicked as a result of γ-irradiation or of Mg^{2+} activation of endogenous nucleases during *Sal* I digestion or represent oligomeric or concatenated circles. The surprising observation that, even after extensive γ-irradiation, the broad band of heterogeneously sized fragments containing *DHFR*-specific sequences migrating near the bottom of the gel in FIGE did not increase in intensity suggests that these fragments may not arise from artifactual degradation of amplisomes during preparation. An interesting possibility is that these fragments are growing DNA chains released from replicative intermediates.

The *DHFR* gene amplification system of HeLa BU25-10B3 cells, described above, exhibits some similarities to that of the extrachromosomal elements of *Leishmania* (3, 22, 23). Whether the first amplisome in 10B3 cells was derived from the chromosomal copy by replication, as has been proposed for the R circles (4) and H circles (22) of *Leishmania*, or by excision, as has been suggested for the *CAD* amplisomes in CHO cells (24), has to be determined.

The present observations suggest that, during development of MTX resistance, there was no appreciable integration of amplisomes into the chromosomes or conversion of amplisomes to DMs. In fact, the latter have been found not to enter the agarose gel during FIGE under the conditions used in the present work (5). The apparent failure of the *DHFR* amplisomes in 10B3 cells to integrate into chromosomes either during the course of gene amplification or during long-term growth of 10B3 cells in the presence of a high concentration of MTX is intriguing and in contrast to the general tendency that has been observed *in vitro* for DMs (23). This property makes such elements potentially useful for the construction of mammalian expression vectors. The failure of the amplisomes to be converted to DMs was confirmed by the finding that no obvious change in their size was observed during 9 months of exposure of 10B3 cells to increasing concentrations of MTX or after a 10-month exposure of 10B3S cells to the maintenance concentration of MTX. The behavior of the *DHFR* amplisomes in 10B3 cells differs in this respect from that which has been reported for the *CAD* amplisomes (24). On the other hand, karyotypic evidence has been obtained (5, 10) that 10B3 cells contain a very small average number of DMs. It is, therefore, possible that these arose by a different mechanism not involving amplisomes. The instability of the *DHFR* amplisomes observed in the absence of selective pressure indicates that these elements are acentromeric, as was shown for the DMs (24) and for the *CAD* amplisomes (8). On the other hand, the finding that the rate of loss of the *DHFR* amplisomes in the absence of selective pressure was much slower than expected from dilution of nonreplicating DNA molecules suggests that they replicate autonomously, as was reported in other systems (6–8).

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